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## Pyridone 6, a Pan-JAK Inhibitor, Ameliorates Allergic Skin Inflammation of NC/Nga Mice via Suppression of Th2 and Enhancement of Th17

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# Pyridone 6, a Pan-JAK Inhibitor, Ameliorates Allergic Skin Inflammation of NC/Nga Mice via Suppression of Th2 and Enhancement of Th17

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Atopic dermatitis (AD) is a common pruritic inflammatory disease triggered by a defective skin barrier and immunodysregulation. AD has been considered a typical example of a Th2 response associated with allergic disease. In the early phases of the disease, symptoms include IgE hyperproduction, eosinophil accumulation, and mast cell activation; in the chronic phase, a Th1-dominant immune response is also observed at the sites of AD skin lesions. The role of IL-17-producing Th (Th17) cells in AD has not been established. In the current study, we found that pyridone 6 (P6), a pan-JAK inhibitor, delayed the onset and reduced the magnitude of skin disease in an AD-like skin-disease model of NC/Nga mice. P6 reduced IFN- $\gamma$  and IL-13, whereas it enhanced IL-17 and IL-22 expression. In vitro, P6 also inhibited both Th1 and Th2 development, whereas it promoted Th17 differentiation from naive T cells when present within a certain range of concentrations. This was probably because P6 strongly inhibited STAT1, STAT5, and STAT6 phosphorylation, whereas STAT3 phosphorylation was less efficiently suppressed by P6 at the same concentration. Furthermore, IL-22 protects keratinocytes from apoptosis induced by IFN- $\gamma$ , and administration of IL-17 and IL-22 partially ameliorated skin diseases in NC/Nga mice. These results suggested that the JAK inhibitor P6 is therapeutic for AD by modulating the balance of Th2 and Th17. *The Journal of Immunology*, 2011, 187: 000–000.

**A**topic dermatitis (AD) is the most common inflammatory skin disease worldwide, and its incidence is still on the rise (1). Its pathogenesis involves a defect in the skin barrier (2), such as that caused by filaggrin mutation, as well as complex interactions of innate and adaptive immune-system components. Intrinsic abnormalities in skin-barrier function allow the invasion of environmental Ags, such as mites and bacteria, which evoke Ag-specific Th2-shifted immune responses. AD has been considered a typical example of Th2-mediated disease because of

its association with IgE hyperproduction, eosinophil infiltration to skin lesions, and mast cell activation. However, in its later phases, high levels of IFN- $\gamma$  and the appearance of Th1 cells have also been observed (3).

Recently, the Th1–Th2 paradigm has been revised, and Th17 cells have been discovered. Th17 cells, a subset of Th cells producing IL-17 and IL-22, play crucial roles in host defense against extracellular bacteria and fungi, as well as in the pathogenesis of various autoimmune and inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis. Their product, IL-17, is deeply involved in eliminating extracellular microbes through neutrophil accumulation and in evoking antibacterial responses, such as the production of antimicrobial proteins and proinflammatory cytokines from epithelial cells. It has not been established whether the involvement of Th17 cells in AD is pathogenic or protective (4, 5). In a steady state, IL-17 and IL-22 are necessary for host defense; at an acute inflammatory site, they promote tissue remodeling, followed by neutrophil accumulation. In a murine asthma model, IL-17 was likewise shown to play two roles: during the establishment of allergic asthma, it is an essential part of Ag sensitization, whereas in sensitized mice it reduces the severity of the allergic response by inhibiting dendritic cells and chemokine synthesis. Moreover, IL-17A was shown to be protective against T cell-mediated intestinal inflammation (6).

Th17 development is regulated by various factors. In particular, the Th1 cytokines IFN- $\gamma$  and IL-2 and the Th2 cytokine IL-4 strongly inhibit Th17 development both in vitro and in vivo (7). The Th17 differentiation of naive T cells is initiated by IL-6 and TGF- $\beta$  (8–10). In addition, IL-23 and IL-21 are thought to be important in the maturation and/or maintenance of Th17 cells (11–13). IL-6, IL-21, and IL-23 activate STAT3, which is thought to be essential for Th17 differentiation (12–16). Finally, IFN- $\gamma$ , IL-2,

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Abbreviations used in this article: AD, atopic dermatitis; BD,  $\beta$ -defensin; C-nano, empty nanoparticle as a negative control; Dfb ointment, ointment containing *Dermaphagoides farinae* body extract; iTreg, inducible regulatory T cell; LN, lymph node; PB, peripheral blood; PLGA, polylactic acid with glycolic acid; PMN, polymorphonuclear neutrophil; P6, pyridone 6; P6-nano, pyridone 6-encapsulated polylactic acid with glycolic acid nanoparticle.

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and IL-4 activate STAT1, STAT5, and STAT6, respectively. In the absence of these STATs, Th17 development is greatly enhanced (17).

Thus, the balance of Th1, Th2, and Th17 development is primarily determined by the cytokine milieu and by cytokine signal transduction, yet it may also be modulated through the selective suppression of JAK kinase activity. The JAKs are a family of nonreceptor protein tyrosine kinases that plays a critical role in cytokine signaling through activating the cytoplasmic latent forms of STATs via tyrosine phosphorylation on a specific tyrosine residue near the Src homology 2 domain (18). IL-6 mostly uses JAK1, whereas IL-2 and IL-4 activate JAK1 and JAK3. IFN- $\gamma$  activates JAK1 and JAK2. Pyridone 6 (P6), a pan-JAK inhibitor, was developed by Merck Research Laboratories (19) and was reported to inhibit the growth of multiple myeloma cells (20). P6 was shown to inhibit kinase by interacting within the ATP-binding cleft of each JAK. P6 was reported to potently inhibit the JAK kinase family, with IC<sub>50</sub> values of 1 nM for JAK2 and TYK2, 5 nM for JAK3, and 15 nM for JAK1, while displaying significantly weaker affinities (130 nM to >10 mM) for other protein tyrosine kinases (19). This imbalance toward JAK specificity may differentially affect Th development. In this study, we discovered that P6 strongly inhibited Th2 and modestly inhibited Th1, whereas it enhanced Th17 development when present within a certain range of concentrations. This is mostly due to strong suppression of the IFN- $\gamma$ /STAT1-, IL-2/STAT5-, and IL-4/STAT6-signaling pathways by P6, which all inhibit Th17 differentiation, as well as modest suppression of IL-6/STAT3, which is essential for Th17 differentiation (8–10).

P6 has not been used in animal models, probably because of its low solubility in water. We overcame this problem by formulating P6 as a polylactic acid with glycolic acid (PLGA) nanoparticle and were able to successfully treat a murine model of AD with it. Surprisingly, we found that P6 enhanced Th17 in vitro and in vivo. We demonstrated that the exogenous administration of IL-17 reduced the severity of the AD model by inducing antimicrobial peptides and promoting keratinocyte survival. These results suggested that the pan-JAK inhibitor P6 is a potent reagent against AD through its regulation of immune responses.

## Materials and Methods

### Preparation of P6 and P6-encapsulated PLGA nanoparticles

P6 was synthesized by Fuji Molecular Planning Company (Yokohama, Japan), according to the procedures described previously (19), and was prepared as a 100-mM stock solution in DMSO. P6-encapsulated PLGA nanoparticles (P6-nano) were generated at Hosokawa Powder Technology Research Institute (Osaka, Japan) using the emulsion-solvent evaporation technique, as previously described (21). In brief, after P6 and PLGA were dissolved in acetone, ethanol was added and then evaporated by polyvinyl alcohol solvent. PLGA consisted of a mixture of polylactic acid with glycolic acid at a ratio of 3:1. Acetone and ethanol were removed, and the PLGA nanoparticles were freeze-dried. Collectively, the resulting nanoparticles contained 4.6% P6. Our analysis revealed that P6 and P6-nano exhibited similar activity for Th differentiation and for suppression of STAT phosphorylation in vitro (Supplemental Fig. 1).

### Murine AD model

NC/Nga mice were purchased from Charles River (Yokohama, Japan) and used at the age of 10–15 wk. A lesion was created on the rostral region of each mouse's back using an electric clipper, and residual hair was depilated with a hair-removal cream under ether anesthesia. Mice were then challenged on the shaved dorsal skin with an ointment containing *Der-matophagoides farinae* body extract (Dfb ointment, Biostir, Kobe, Japan) as a mite Ag. As a second Ag challenge, the mouse skin barrier was disrupted through treatment with 150  $\mu$ l 4% SDS before Dfb ointment was applied to the dorsal skin, and SDS/Dfb ointment treatments were repeated twice a week for ~30 d after the first mite Ag challenge.

### P6-encapsulated PLGA nanoparticles, betamethasone, and IL-17 treatment

To assess the effect of P6 treatment on AD symptoms, nanoparticles containing P6 (2 mg/body) or empty nanoparticles as a negative control (C-nano) were dissolved in 0.1 ml saline and administered s.c. 1 d after Dfb ointment application; this treatment was repeated twice a week. To assess the effects of recombinant murine IL-17 and IL-22, these cytokines (50  $\mu$ g/kg) or 100  $\mu$ l PBS was administered for the same duration as the nanoparticles. Twenty milligrams of 0.064% betamethasone ointment (Shionogi, Japan) were applied to the dorsal lesion of mice once a week.

### Scoring of dermatitis and immunohistochemical and histological observation

Each of several factors—erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion—was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of these individual scores was taken as the overall dermatitis score (22).

Portions of the dorsal skin were fixed with 4% paraformaldehyde in saline, embedded in paraffin, and sectioned into 8- $\mu$ m-thick slices, which were stained with H&E. For immunohistochemistry in Supplemental Fig. 3, the fixed sections were processed using the standard HRP-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) procedure.

### Measurement of cytokines and serum Igs

For cytokine measurement and serum Igs, we used ELISA kits (eBioscience, San Diego, CA and Bethyl Laboratory, Montgomery, TX, respectively), according to the manufacturers' instructions. The peripheral blood (PB) of each mouse was collected from the retro-orbital sinus at the indicated time points, and sera were prepared.

### Real-time RT-PCR

Real-time PCR analysis was performed using epidermis of pinnae freshly harvested from AD mice. After harvesting, the epidermis was washed with cold PBS and submerged on RNeasy (Qiagen, Valencia, CA) overnight. Then, the epidermis was minced and homogenized in lysis buffer in an RNeasy kit (Qiagen). Total RNA was isolated from the epidermis, according to the manufacturer's protocol, and reverse transcribed using Revvanta Ace (Toyobo). Real-time PCR was performed using Applied Biosystems 7300 real-time PCR system with SYBR Green PCR Master Mix (Applied Biosystems) (23) and the primers listed in Table I. The data were normalized to  $\beta$ -actin, chosen as an endogenous control.

### T cell differentiation in vitro

Favorable conditions for the differentiation of the various Th cell subsets were created through stimulation with the following cytokines: plate-bound anti-CD3 and 10  $\mu$ g/ml anti-IL-4 (11B11; eBioscience) and 10  $\mu$ g/ml anti-IFN- $\gamma$  (R4-6A2; eBioscience) for Th0 (neutral conditions); 10  $\mu$ g/ml anti-IL-4 and 10 ng/ml IL-12 (PeproTech, London, U.K.) for Th1; 10  $\mu$ g/ml anti-IFN- $\gamma$  and 10 ng/ml IL-4 (PeproTech) for Th2; 10  $\mu$ g/ml anti-IL-4, 10  $\mu$ g/ml anti-IFN- $\gamma$ , and 2 ng/ml TGF- $\beta$  (R&D Systems, Minneapolis, MN) for inducible regulatory T cell (iTreg); 10  $\mu$ g/ml anti-IL-4, 10  $\mu$ g/ml anti-IFN- $\gamma$ , 20 ng/ml IL-6 (PeproTech), 2 ng/ml TGF- $\beta$  for Th17 (24). Activated cells were restimulated with 50 ng/ml PMA (Calbiochem, San Diego, CA) and 0.5  $\mu$ g/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A (eBioscience) for 4 h before intracellular staining or were stimulated with 1  $\mu$ g/ml anti-CD3 for 1 d before ELISA. Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA), as previously described (25).

### Flow cytometric analysis

Levels of IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>, IL-17<sup>+</sup>, and Foxp3<sup>+</sup> cells in the various Th subsets were measured by flow cytometry, as previously described (26). After surface staining with CD4-PerCP Cy5.5 with/without CD25-PE, cells were washed and incubated with unlabeled anti-CD16/32 mAb for 20 min to prevent nonspecific binding via Fc $\gamma$ RI. The cells were suspended in Fixation Buffer, and intracellular cytokine staining was performed with permeabilized buffer (eBioscience), according to the manufacturer's instructions. A FACSCanto instrument, in conjunction with Diva software (BD Biosciences), was used to detect each intracellular cytokine<sup>+</sup> T cell subset. A total of 50,000 cells was acquired, and an electronic gate was set on lymphocytes using the forward and side scatter plots. CD4<sup>+</sup> T cells falling within the gated area were identified for the detection of each cytokine.

Western blotting for various STAT proteins and phosphorylated STATs

Naive CD4<sup>+</sup> T cells were treated with various concentrations of P6 in RPMI 1640 medium (Life Technologies, Rockville, MD) 1 h before the

appropriate cytokines were added to create each Th-differentiating condition. Immunoblotting was performed using antiphospho-STAT protein Abs (Cell Signaling Technology, Danvers, MA) or anti-total STAT protein Abs (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (27).

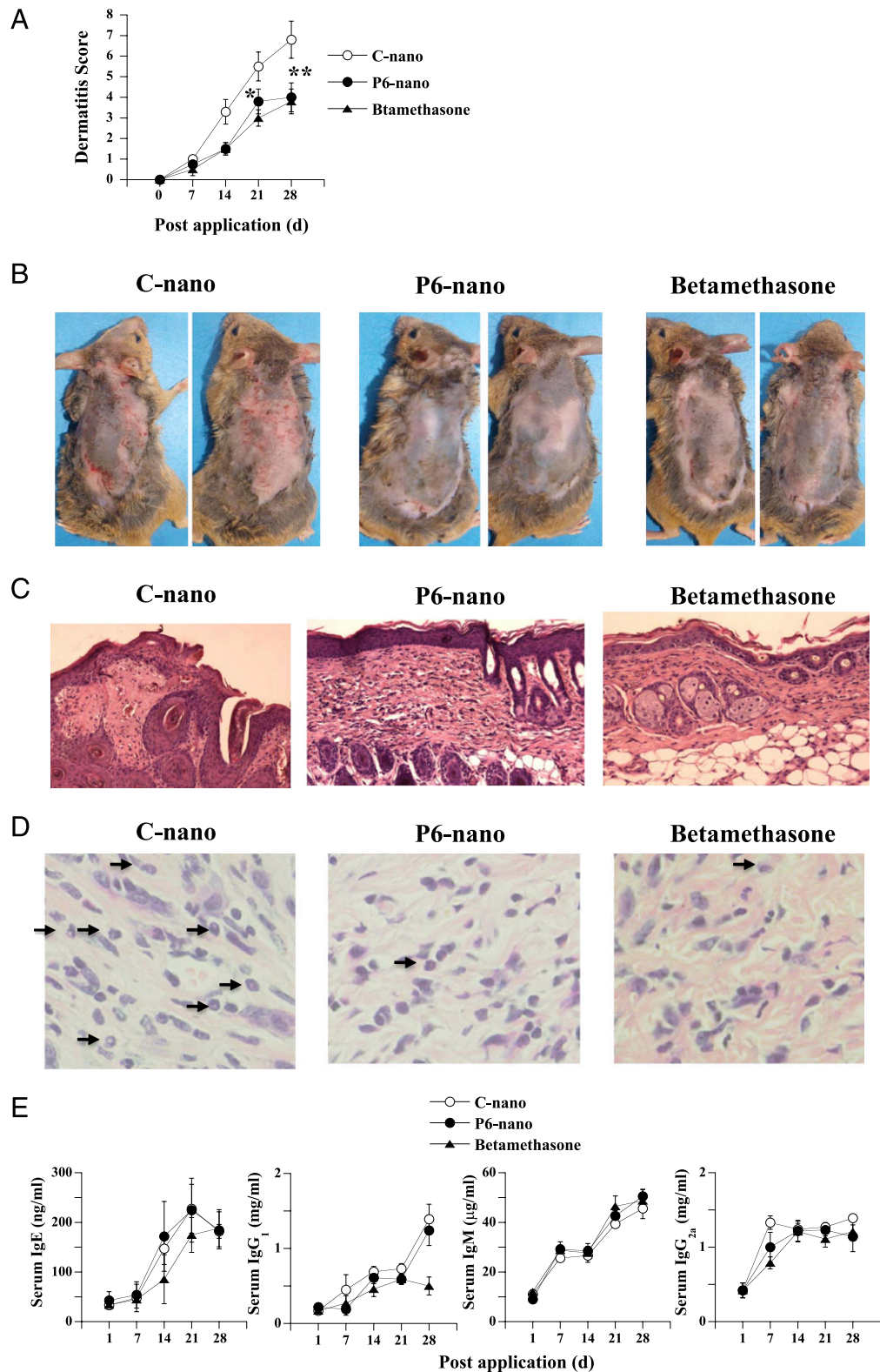


**FIGURE 1.** P6 treatment of AD in NC/Nga mice. Development of allergic skin inflammation due to Dfb ointment application on C-nano-, P6-nano-, or betamethasone-treated mice. C-nano and P6-nano were administered s.c. twice a week, always 1 d after Dfb application. *A*, Dermatitis scores were assessed at the indicated time points. \* $p < 0.05$ , \*\* $p < 0.01$ , P6-nano-treated mice versus C-nano-treated mice. *B*, Skin features of each mouse at 30 d after first Dfb application. *C*, Histological changes of dorsal skin from each mouse. Original magnification  $\times 20$ . *D*, Typical histological observation of dorsal skin. H&E, original magnification  $\times 40$ . *E*, Serum Ig levels were evaluated at the indicated time points. All data represent mean  $\pm$  SD ( $n = 5-8$  mice/group).

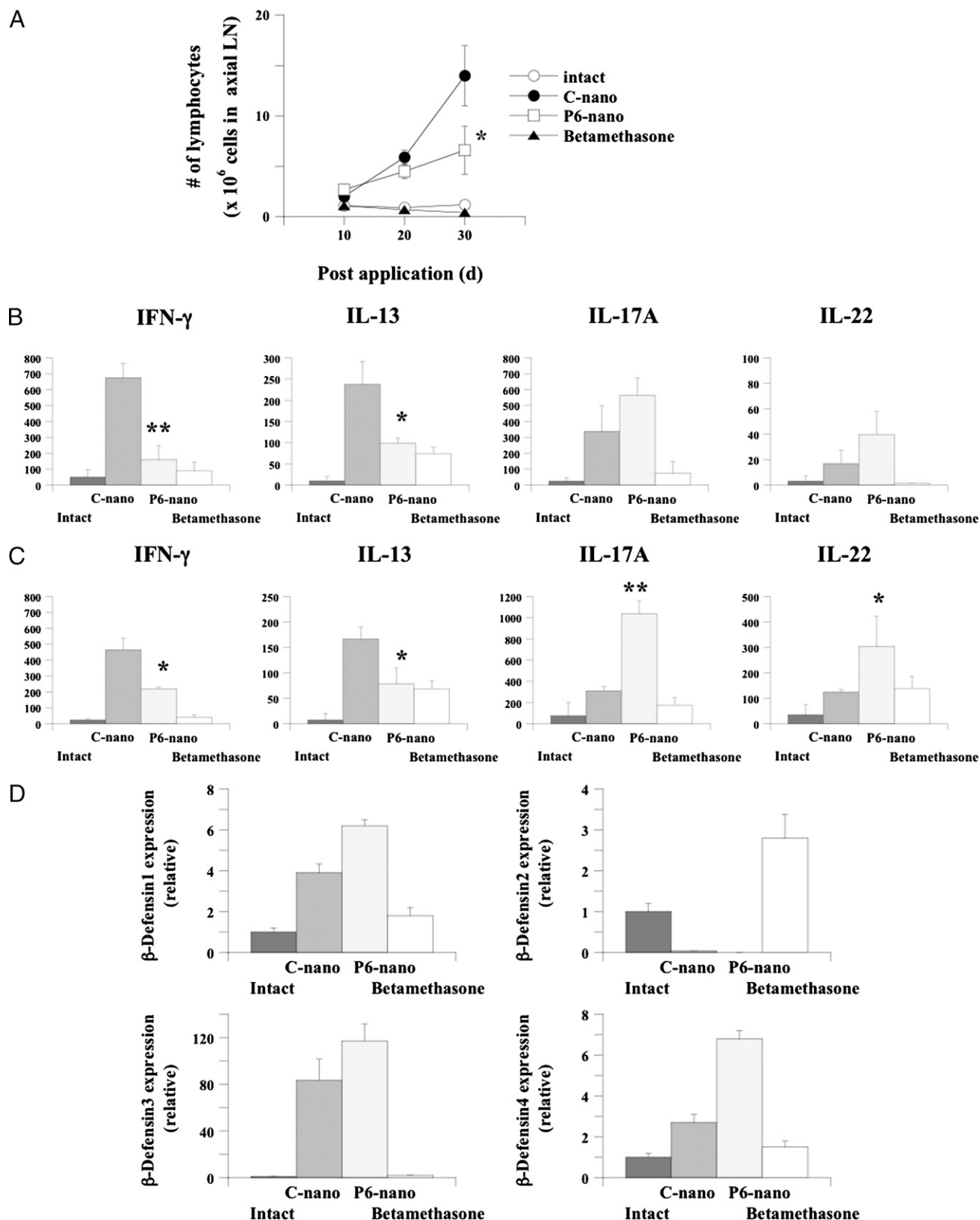


**FIGURE 2.** The regulatory effect of P6-nano on proliferation and cytokine production by lymphocytes. **A**, Axial LN were removed, and single-cell suspensions were prepared at the indicated time points. Data represent mean  $\pm$  SD ( $n = 5$  mice/group). Lymphocytes from LN of each group of mice were prepared 10 d (**B**) or 4 wk (**C**) after the first Dfb ointment application and were stimulated with soluble anti-CD3 Ab (1  $\mu$ g/ml) for 72 h. Supernatants were collected, and cytokine concentrations were measured (y-axis; pg/ml). Data represent mean  $\pm$  SD from triplicate experiments. **D**, Total RNA was isolated from the skin lesions of intact or drug-treated mice, and BD mRNAs were measured by real-time PCR 4 wk after onset of the disease. Values are presented relative to baseline expression in epidermal tissues from intact mice (set to 1). \* $p < 0.05$ , \*\* $p < 0.05$ , P6-nano–treated mice versus C-nano–treated mice.

Table I. Primer pairs used for real-time PCR analysis

| Gene       | Forward Primer                           | Reverse Primer                              |
|------------|------------------------------------------|---------------------------------------------|
| b-actin    | 5'-GAT TAC TGC TCT GGC TCC TAG C-3'      | 5'-GAC TCA TCG TAC TCC TGC TTG C-3'         |
| BD 1       | 5'-GGC ATT CTC ACA AGT CTT GGA CGA AG-3' | 5'-AGC TCT TAC AAC AGT TGG GCT TAT CTG G-3' |
| BD 2       | 5'-TCT CTG CTC TCT GCT GCT GAT ATG C-3'  | 5'-AGG ACA AAT GGC TCT GAC ACA GTA CC-3'    |
| BD 3       | 5'-CAC GAG GCA CCA GGC TTC AGT C-3'      | 5'-CAA TGG GAT GAA CAG AAT TTG CTC C-3'     |
| BD 4       | 5'-GCA GCC TTA CCC AAA TTA TC-3'         | 5'-ACA ATT GCC AAT CTG TCG AA-3'            |
| Loricrin   | 5'-CCT GTG GGT TGT GGA AAG ACC-3'        | 5'-AGA GCC TCC TCC AGA TGA GC-3'            |
| Involucrin | 5'-AGA ACT GCA TCT GGG TCA GC-3'         | 5'-GCT TCT GCT TCT GTT TCT GTC C-3'         |
| S100a8     | 5'-CCT TGC GAT GGT GAT AAA AGT G-3'      | 5'-CCC AGC CCT AGG CCA GAA-3'               |
| Keratin6   | 5'-TTC TCT ACT TCC CAG CCT TCT CA-3'     | 5'-GCC ACG GTG GCT GGT TT-3'                |

### Statistical analysis

All data were analyzed by the Student *t* test. A *p* value < 0.05 was considered significant. All data are expressed as mean ± SD.

## Results

### P6-nano treatment ameliorates AD

NC/Nga mice were challenged with Dfb ointment containing mite extract on the dorsal lesion; 2 wk later, these mice gradually began to develop symptoms of AD, including dryness, edema, and hemorrhage (28). Because P6 alone was difficult to dissolve in PBS at high concentrations, we developed P6-nano. P6-nano strongly ameliorated AD in NC/Nga mice, exerting an effect comparable to that of betamethasone ointment, a commonly used drug, which we also tested as a positive control (Fig. 1A). In contrast, empty PLGA nanoparticles (C-nano) seemed to have no effect.

As shown in Fig. 1B, gross examination of P6-nano-treated mice revealed less edema and hemorrhage on their backs compared with C-nano-treated mice. In the skin lesions of control mice, H&E staining revealed massive infiltration of mononuclear cells and eosinophils, hyperplasia, and spongiosis of keratinocytes due to widespread apoptosis; in contrast, P6-nano-treated mice did not develop these symptoms (Fig. 1C, 1D). Serum Ig levels increased gradually and were not significantly different between the two groups, whereas the IgG1 concentration of betamethasone-treated mice was suppressed (Fig. 1E). Therefore, P6 administration on skin lesions did not affect systemic Ig production, which suggested that the AD-like skin disorder in NC/Nga mice does not depend on the serum IgE, as reported previously (29).

### P6-nano treatment prevents Th1 and Th2 cytokine production but upregulates IL-17 and IL-22 production in axillary lymph nodes

The numbers of lymphocytes in the axillary lymph nodes (LN) of AD mice increased gradually as dermatitis developed. Lymphocyte counts remained lower in P6-nano-treated mice than in control mice (Fig. 2A), but lymphocyte proliferation in the LN was significantly higher in P6-nano-treated mice than in betamethasone-treated mice or intact mice. Moreover, the pattern of cytokine production was modified by P6 treatment. In control Dfb ointment-challenged mice, IFN- $\gamma$  and IL-13 levels were elevated 10 d after application (acute phase, Fig. 2B), as well as 4 wk later (chronic phase, Fig. 2C); in contrast, the levels of these cytokines were reduced in P6-nano- or betamethasone-treated mice. Interestingly, IL-17 and IL-22 production was significantly enhanced by P6-nano treatment, especially in the later phase, but it was suppressed by betamethasone treatment. The suppression of cytokine production that occurs as a result of betamethasone treatment may be due to a strong inhibition of LN cell expansion (Fig. 2A). However, P6-nano suppressed IFN- $\gamma$  and IL-13 production but enhanced IL-17 and IL-22 production in LN cells,

because LN cells expanded in both the acute and chronic phases of AD. Thus P6-nano seems to modulate Th differentiation in this *in vivo* AD model.

IL-17 and IL-22 from Th17 cells have been thought to promote the production of  $\beta$ -defensins (BD) from keratinocytes (30, 31). As shown in Fig. 2D, P6-nano treatment augmented BD-1, -3, and

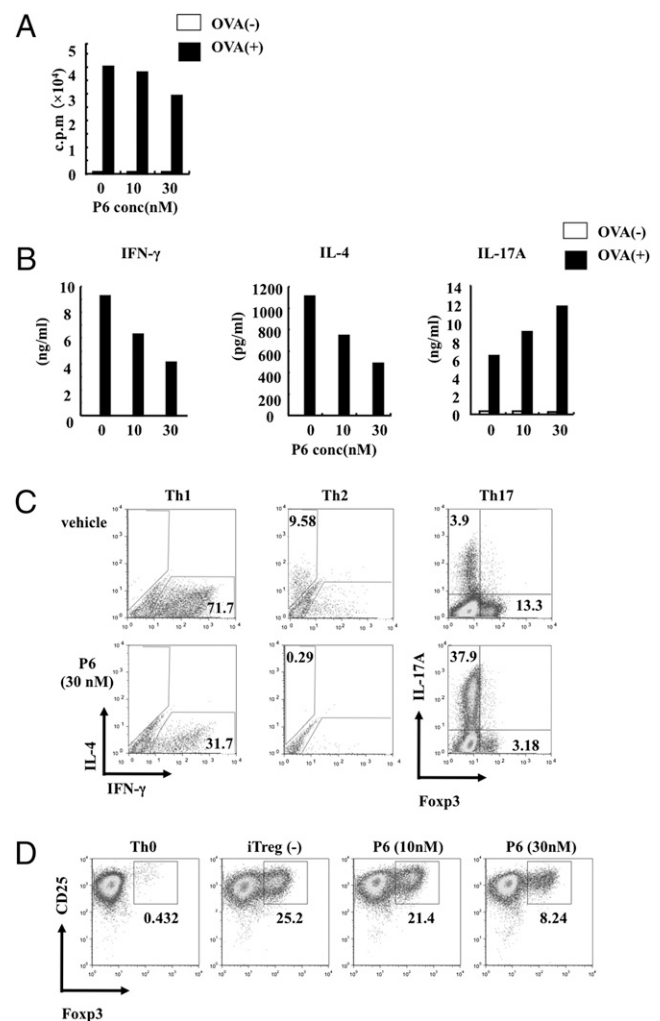


**FIGURE 3.** P6 modified naive CD4<sup>+</sup> T cell differentiation. Proliferation (A) and cytokine production (B) of splenocytes from OT-II mice stimulated with 100 ng/ml OVA at the indicated P6 concentrations. C, The various conditions at which Th cells were stimulated with 30 nM of P6 or vehicle for 3 or 5 d, as described in *Materials and Methods*. Th cells were restimulated with PMA and ionomycin, and their intracellular cytokine production was measured. D, Foxp3 staining was performed using an iTreg preparation. Data are from one representative of three independent experiments with two OT-II or B6 mice and are depicted as mean ± SD of triplicate in A and B.

-4 expression compared with C-nano treatment. In contrast, gene expression of epidermal differentiation complex, such as S100a8, involucrin, loricrin, and keratin, were not very different between C-nano- and P6-nano-treated mice (Supplemental Fig. 3), indicating that P6-nano treatment did not affect keratinocyte differentiation. The specific primer sequences used in reactions were listed in Table I.

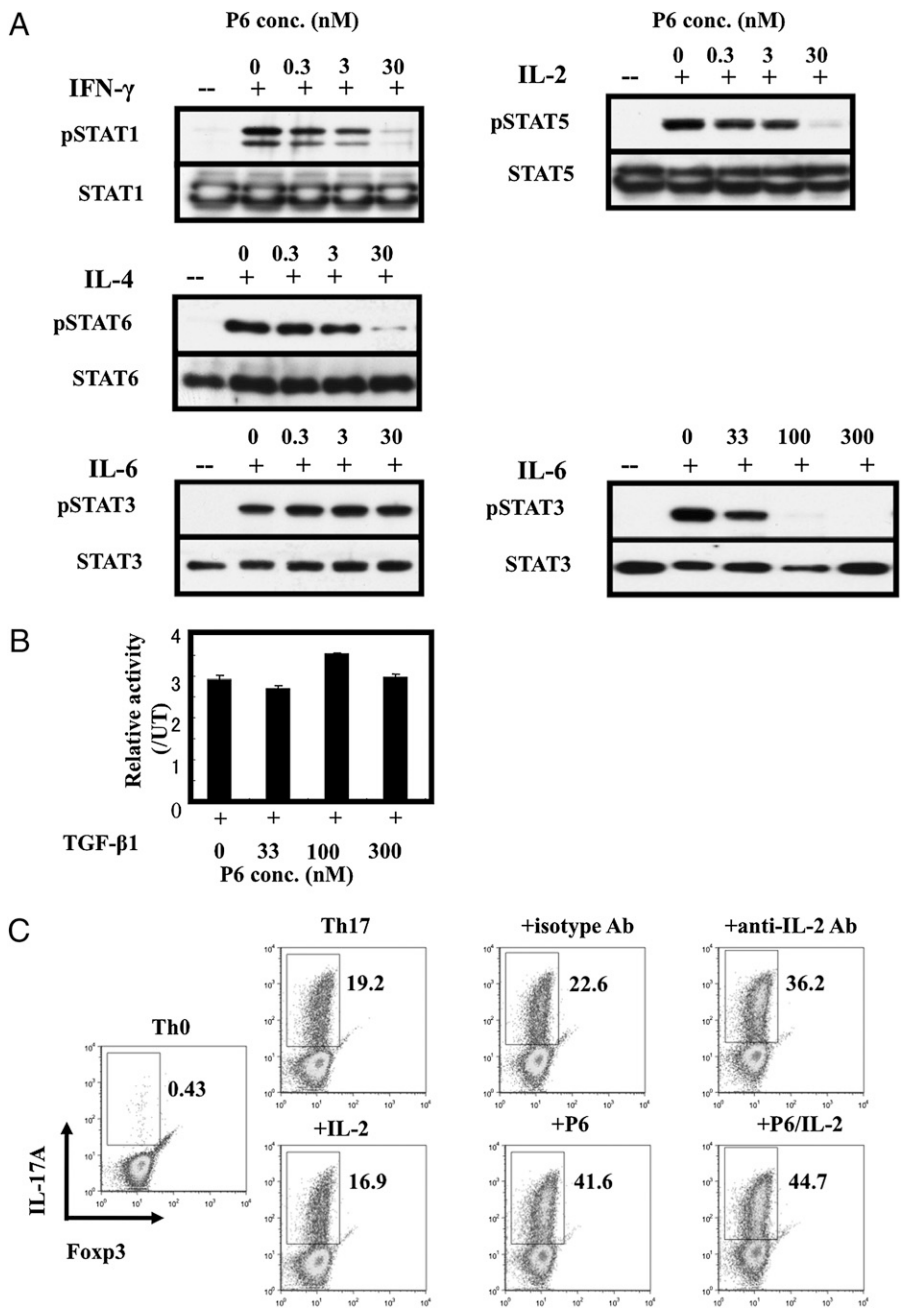
*P6 inhibits Th1/2 but enhances Th17 differentiation from naive CD4<sup>+</sup> T cells in vitro*

P6 treatment affected the numbers of LN cells, but it made no significant difference in serum IgE levels, suggesting that P6 might modify the function of T cells rather than B cells. To assess the effect of P6 on CD4<sup>+</sup> T cell activation and differentiation, we administered it to naive CD4<sup>+</sup> T cells from OVA-specific OT-II or B6 mice, cultured under the Th1, Th2, Th17, or regulatory T cell condition, and determined the resulting cytokine production pro-

file by means of ELISA and intracellular cytokine staining. As shown in Fig. 3A and 3B, at concentrations at which P6 had little effect on proliferation, it nevertheless inhibited IFN- $\gamma$  and IL-4 production and enhanced the production of IL-17 by OT-II T cells in response to 100 ng/ml of OVA. These results were confirmed by intracellular cytokine staining (Fig. 3C). P6 inhibited Th1 and Th2 differentiation, but it strongly enhanced Th17 differentiation. In addition, TGF- $\beta$  plus IL-2 induced Foxp3 expression in naive T cells (iTreg). As shown in Fig. 3D, iTreg differentiation was apparently inhibited by P6, probably as a result of the suppression of IL-2 signaling by P6.

Next, we investigated the mechanisms underlying the differential effects of P6 on Th differentiation. First, we identified the proteins that were phosphorylated when naive CD4<sup>+</sup> T cells were stimulated with IFN- $\gamma$ , IL-2, IL-4, or IL-6. As shown in Fig. 4A, IFN- $\gamma$ -mediated STAT1 phosphorylation, IL-2-mediated STAT5 phosphorylation, and IL-6-mediated STAT6 phosphorylation were

**FIGURE 4.** P6 regulated various cytokine signaling pathways in naive CD4<sup>+</sup> T cells. **A**, Naive CD4<sup>+</sup> T cells were cultured with plate-bound anti-CD3 Ab and the indicated cytokine. One hour later, cells were collected, and a cytosolic fraction was prepared for an immunoblotting assay. **B**, HEK293 cells were transfected with a luciferase reporter construct controlled by a Smad-binding element. Luciferase activities are expressed as fold-increase over the background without TGF- $\beta$ . **C**, Naive CD4<sup>+</sup> T cells were cultured in Th0 or Th17 condition in the absence or presence of anti-IL-2 or isotype Ab (10  $\mu$ g/ml) or in the presence IL-2 (10 ng/ml). Data are representative of three independent experiments.



equally inhibited by the indicated concentrations of P6. The  $IC_{50}$  of P6 was  $\sim 3$  nM for all of these cytokines; this is comparable to the reported  $IC_{50}$  values of P6 for JAK2, Tyk2, and JAK3. Interestingly, only STAT3 phosphorylation by IL-6 stimulation was not completely abrogated by P6 at a concentration of 30 nM; the complete inhibition of STAT3 phosphorylation required 100 nM of P6 (Fig. 4A). This may be due to the relatively low  $IC_{50}$  of P6 for JAK1. P6 had a weak effect on TGF- $\beta$ -mediated Smad-transcriptional activity (Fig. 4B).

We suspected that the variation in the ability of P6 to suppress these different types of STAT phosphorylation was responsible for the variation in Th differentiation by P6. It was reported that STAT3 promotes Th17 differentiation, whereas STAT1, STAT5, and STAT6 inhibit it (32, 33). Therefore, at concentrations ranging from 10–30 nM, P6 promoted Th17 differentiation by suppressing STAT1, STAT5, and STAT6 without suppressing STAT3 or TGF- $\beta$  signaling. This was confirmed by our observation of Th17 differentiation in the presence of IL-2 or anti-IL-2 Ab. IL-2 suppressed Th17 differentiation, but P6 did not; rather, it increased the Th17 population similarly to IL-2-neutralizing Ab (Fig. 4C). Therefore, the differential effect of P6 on IL-6/STAT3 and IL-2/STAT5 is a plausible mechanism for the promotion of Th17 differentiation by P6.

We assessed the effects of P6-nano on STAT phosphorylation in skin lesions of NC/Nga mice using immunohistochemistry. Phosphorylated STAT1 and STAT3 were successfully detected (Supplemental Fig. 2A, 2B, respectively). Leukocytes infiltrated skin lesions showed strong STAT1 phosphorylation in C-nano-treated mice, whereas STAT1 phosphorylation was weaker in P6-nano- or betamethasone-treated mice (Supplemental Fig. 2A). STAT3

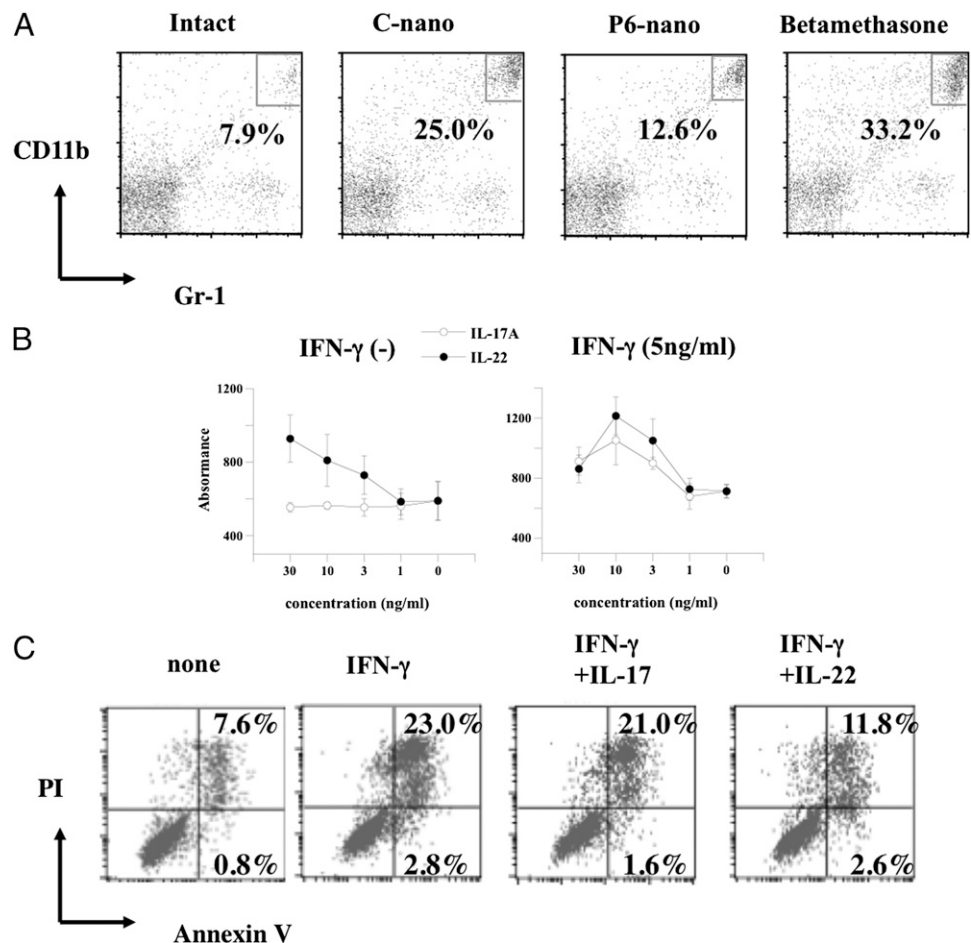
phosphorylation was mostly detected in hair follicle keratinocytes, and P6-nano-treated mice exhibited increased intensity of STAT3 phosphorylation, reflecting higher levels of IL-22 (34) (Supplemental Fig. 2B). These data are consistent with our hypothesis that P6 suppresses Th1 and Th2 but enhances Th17.

#### *IL-17 and IL-22 protects keratinocytes from IFN- $\gamma$ -induced apoptosis*

Knowing that P6 treatment enhanced Th17 cell development both in vitro and in vivo, we tried to clarify the role of Th17 cells in AD. IL-17 is known to accumulate neutrophils at the sites of inflammatory lesions, yet P6-nano-treated mice did not exhibit excessive neutrophil infiltration into their skin lesions or into circulation in the PB. The population of neutrophils was actually lower in P6-nano-treated mice than in C-nano-treated mice (Fig. 5A).

In the chronic phase of AD, IFN- $\gamma$  production was predominant in skin lesions, where it resulted in keratinocyte apoptosis (35), causing skin barrier dysfunctions, such as spongiosis (Fig. 1D). Accordingly, we conducted experiments using the human keratinocyte cell line HaCaT to investigate whether IL-17 or IL-22 could protect keratinocytes from apoptosis induced by IFN- $\gamma$ . We observed that both IL-17 and IL-22 enhanced the proliferative activity of HaCaT cells in the presence of added IFN- $\gamma$ . In response to IL-22, HaCaT cells showed increased proliferative activity in a dose-dependent manner, regardless of whether IFN- $\gamma$  was added, but IL-17 alone could not affect HaCaT cell proliferation without IFN- $\gamma$  (Fig. 5B). The precise mechanism by which the combination of IL-17 and IFN- $\gamma$  induces proliferative activity of HaCaT cells is unknown. However, it is known that

**FIGURE 5.** Role of IL-17 and IL-22 in protecting keratinocytes from apoptosis. **A**, PBLs were prepared from three mice in each group 30 d after the first Dfb ointment application, and PMN (CD11b<sup>+</sup>Gr-1<sup>+</sup>) were analyzed by flow cytometry. All mice in each group showed similar results; typical results are shown. **B**, A total of  $4 \times 10^3$  HaCaT cells was cultured in 2% FCS containing RPMI 1640 with the indicated concentrations of IL-17A or IL-22. Twenty-four hours later, 5 ng/ml of IFN- $\gamma$  was added, and the cells were cultured for an additional 2 d. Proliferation activities were measured using water soluble tetrazolium-8 reagent and a microplate reader. **C**, HaCaT cells were cultured with 10 ng/ml of IL-17 or IL-22, and the procedure described above was continued. HaCaT cells were stained with propidium iodide and FITC-labeled Annexin V, and flow cytometric analysis was performed. Experiments were repeated three times with similar results.



keratinocytes undergo apoptosis when IFN- $\gamma$  induces FasL expression (36). When IL-22 does not affect the expression of FasL induced by IFN- $\gamma$  in keratinocytes, the numbers of Annexin V<sup>+</sup> cells were reduced (Fig. 5C), suggesting that IL-22 interrupts the death signal through a Fas-FasL interaction involving an anti-apoptotic molecule, such as Bcl-2 (37).

These results supported the hypothesis that the production of Th17 cells in AD patients is required to improve the symptoms of AD, because these cells support the maintenance of keratinocytes and their antimicrobial defenses (5).

#### *Exogenous IL-17 and IL-22 administration ameliorates AD symptoms*

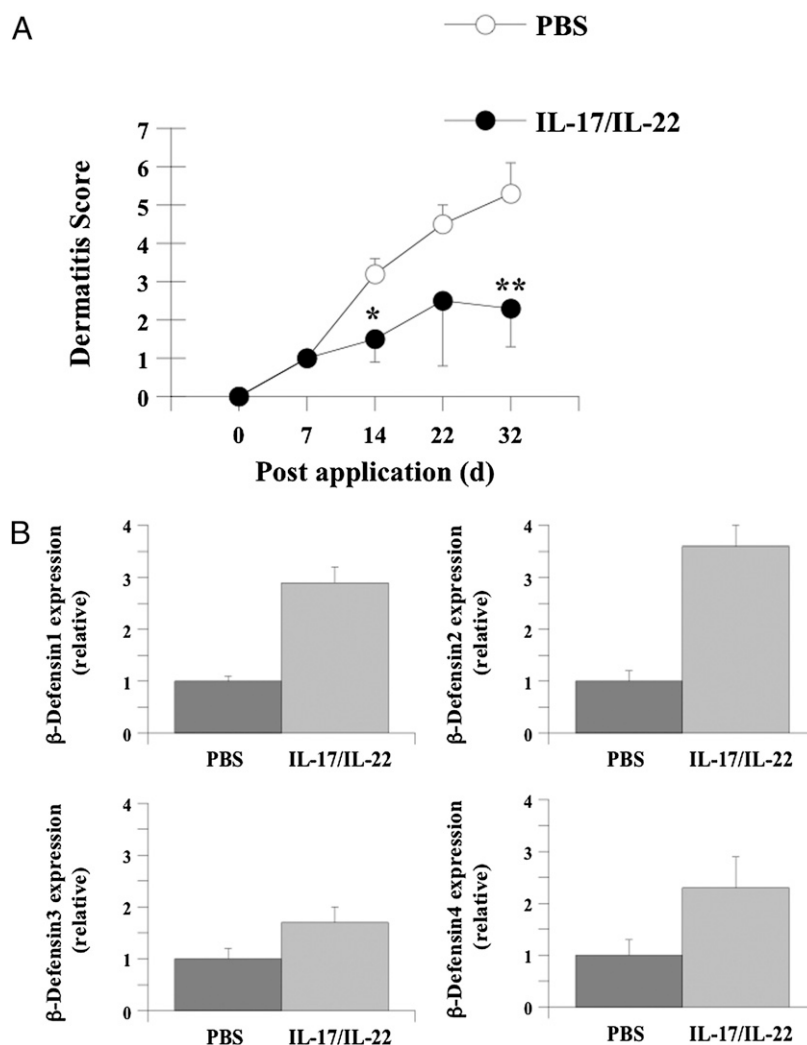
P6 treatment stimulates the production of Th17 cells, which can produce both IL-17 and IL-22; accordingly, we sought to determine whether IL-17 and IL-22 could modulate AD symptoms. Because lymphocytes in the draining LN of AD skin lesions can produce small amounts of IL-17 and IL-22, these cytokines were thought to be involved in the immunopathogenesis of AD (4). We examined the therapeutic effects of rIL-17 and rIL-22 based on the observation that P6-nano-treated AD mice had higher levels of these cytokines than did C-nano-treated AD mice. As expected, treatment with these cytokines ameliorated AD symptoms (Fig. 6A), although no differences in serum IgE concentration were seen between PBS- and cytokine-treated mice (data not shown). The skin lesions of cytokine-treated mice exhibited upregulated expression of antimicrobial proteins, such as BD (Fig. 6B), and

antiapoptotic molecules, including Bcl-2 and Bcl-xL (data not shown). These results indicated that the administration of exogenous IL-17 and IL-22 inhibited the progression of AD symptoms by protecting keratinocytes and evoking antimicrobial activity, whose actions help to maintain skin barrier function. Most AD patients suffer from significant *Staphylococcus aureus* colonization after the loss of their antimicrobial immunity, including proper keratinocyte function and the ability to accumulate neutrophils at AD lesions. Thus, IL-17 and IL-22 production at AD skin lesions could ameliorate AD symptoms. Our results demonstrated that the application of exogenous IL-17 and IL-22 and the induction of Th17 at AD skin lesions do not contribute to pathogenesis but rather are potent reagents for AD therapy.

## Discussion

We showed that the pan-JAK inhibitor P6 possesses therapeutic activity against AD by modifying Th cell differentiation and suppressing mast cell function. P6 and other JAK inhibitors were developed as drugs for myeloproliferative diseases and leukemias caused by mutated JAKs; the JAK kinase inhibitor CP-690550 (tasocitinib; developed by Pfizer, New York, NY) is now in phase II/III clinical trials for rheumatoid arthritis treatment, and it has been shown to be very effective. In this study, we demonstrated that the pan-JAK inhibitor P6 is effective against allergic diseases, such as AD, although AD is not related to JAK mutation. In AD, interactions between various cytokines in a complex

**FIGURE 6.** Exogenous IL-17 and IL-22 administration ameliorates allergic skin inflammation. **A**, One microgram/body each of IL-17 and IL-22 were administered s.c. twice a week to NC/Nga mice. Mice receiving this treatment had lower dermatitis scores, including lower scores for edema and hemorrhage resulting from allergic skin inflammation. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with PBS-treated mice. **B**, Thirty-five days after the first Dfb ointment application, pinnae of PBS- or IL-17 and IL-22-treated mice were removed, and total RNA was prepared for real-time RT-PCR. Results are presented relative to baseline expression in epidermal tissues from PBS-treated mice, set to 1. All data represent mean  $\pm$  SD of five mice/group.



symptom lead to exacerbation to the severe and chronic stages of the disease. As we reported previously, P6 suppresses an IL-13-mediated asthma model (38), suggesting that the modification of cytokine signals is a reasonable strategy for the treatment of immunological disorders. Because each of the current JAK inhibitors has a different specificity for a particular JAK, different JAK inhibitors may have different effects on Th differentiation and, thus, on different immune diseases.

In this study, we showed that increased levels of IL-17- and IL-22-producing CD4<sup>+</sup> T (Th17) cells were required for P6 to induce a series of therapeutic responses to AD. The role of Th17 in AD has not been clarified; however, it was been reported with regard to psoriasis that the expression levels of both IL-23 and IL-17 were very low and that some of the proteins involved in innate defense were also present at abnormally low levels (5, 39). Although the functions of IL-17 and IL-22 in AD remain controversial, it is likely that they are somehow involved. Given that psoriasis and AD share many pathogenic features, including epithelial hyperplasia and the prominent infiltration of immune cells, it seems likely that skin inflammation could be elicited in AD by a mechanism similar to that which occurs in psoriasis. Because the Th17-IL-23 axis plays a critical role in the pathogenesis of psoriasis (40, 41), it could also be involved in AD. The most significant difference between psoriasis and AD is that AD patients exhibit altered skin barrier function, which makes them more susceptible to infection (42). One of the key pathogenic events in AD is the exacerbation of skin inflammation due to spongiosis, which is caused by keratinocyte apoptosis (43). Therefore, we investigated the roles of IL-17 and IL-22 in keratinocyte apoptosis when induced by IFN- $\gamma$ , which is a dominant cytokine during the chronic phase of AD. As shown in Fig. 5, keratinocytes are protected from apoptosis and stimulated to proliferate by the presence of IL-17 and IL-22. Although IL-17 could not completely inhibit the apoptosis induced by IFN- $\gamma$ , the number of viable keratinocytes was visibly increased by its presence. At the site of an inflammatory lesion, IL-17 and IFN- $\gamma$  signaling might function synergistically, perhaps in an upregulating cell cycle. In contrast, IL-22 could inhibit apoptosis by inducing antiapoptotic molecules, such as Bcl2 and Bcl-xL (44). These results indicated that an appropriate milieu of cytokines, including IL-17 and IL-22, is required to protect the skin barrier in AD.

Th17 cells are involved in the pathogenesis of several autoimmune and inflammatory diseases but are also required to protect the host against infection and to control mucosal bacterial flora (45). Furthermore, IL-17 could evoke antibacterial immunity in the skin against such pathogens as *S. aureus* (46). Most AD patients are also infected with *S. aureus* at the sites of inflammatory skin lesions (47) because they lack the innate immunity provided by polymorphonuclear neutrophils (PMN) (48) and NK cells (49). In turn, cutaneous *S. aureus* infection exacerbates the skin inflammation associated with AD. Indeed, PMN in the PB of C-nano- and betamethasone-treated mice contained lower levels of CD49b, an integrin used for homing to inflammatory lesions, as well as lower levels of reactive oxygen species, which play a critical role in attacking bacteria, than did PMN from P6-nano-treated mice (data not shown). As Fig. 5A shows, the PMN populations in the PB of C-nano- and betamethasone-treated mice were robustly elevated; in contrast, the PMN populations in P6-nano-treated mice were only slightly higher than those in intact mice, suggesting that the PMN of mice with severe AD might circulate in the PB rather than accumulate in lesion sites because they have lower levels of integrin expression.

Betamethasone treatment resulted in a superior therapeutic effect, but its long-term use eventually results in immunosuppression;

repeated treatments can make the host more susceptible to *S. aureus* infection. Therefore, the JAK inhibitor P6, which does not suppress immunity, could offer an advantage over betamethasone as a treatment for AD. Indeed, the PMN of P6-nano-treated mice showed higher reactive oxygen species expression levels than did those of betamethasone-treated mice. IL-17 and IL-22 also promote antimicrobial peptides, such as BD. Thus, P6 treatment may contribute to a more efficient elimination of extracellular pathogens, such as antibacterial activity by BD and PMN.

AD has been considered a Th2-mediated disease, but the cytokine milieu at the site of an AD skin lesion is complicated, as shown in Fig. 2B and 2C. In the early phase of AD, Th1 and Th2 production is dominant, whereas IL-17 and IL-22 production is gradually increasing. Therefore, IL-17 and IL-22 have been thought to contribute to the onset of allergic inflammation. A recent report supporting this idea showed that augmented IL-17 production evoked a potent inflammatory response to keratinocytes that included IL-8 and chemokine production, and this response contributed to the exacerbation of AD; however, it should be noted that an unnatural and excessive dose of exogenous IL-17 was administered against keratinocytes in that study (4). Under more ordinary circumstances, IL-17 production is lower in AD patients than in psoriasis patients; specifically, less IL-17 is secreted (50). In addition, our results suggested that IL-17 and IL-22 could reduce keratinocyte apoptosis and support the maintenance of the skin barrier at AD skin lesion sites. The JAK inhibitor P6, which increases the concentrations of IL-17 and IL-22, is an effective therapeutic reagent against AD, augmenting Th17 differentiation and downregulating Th1, Th2, and mast cell activation at AD skin lesion sites.

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## Disclosures

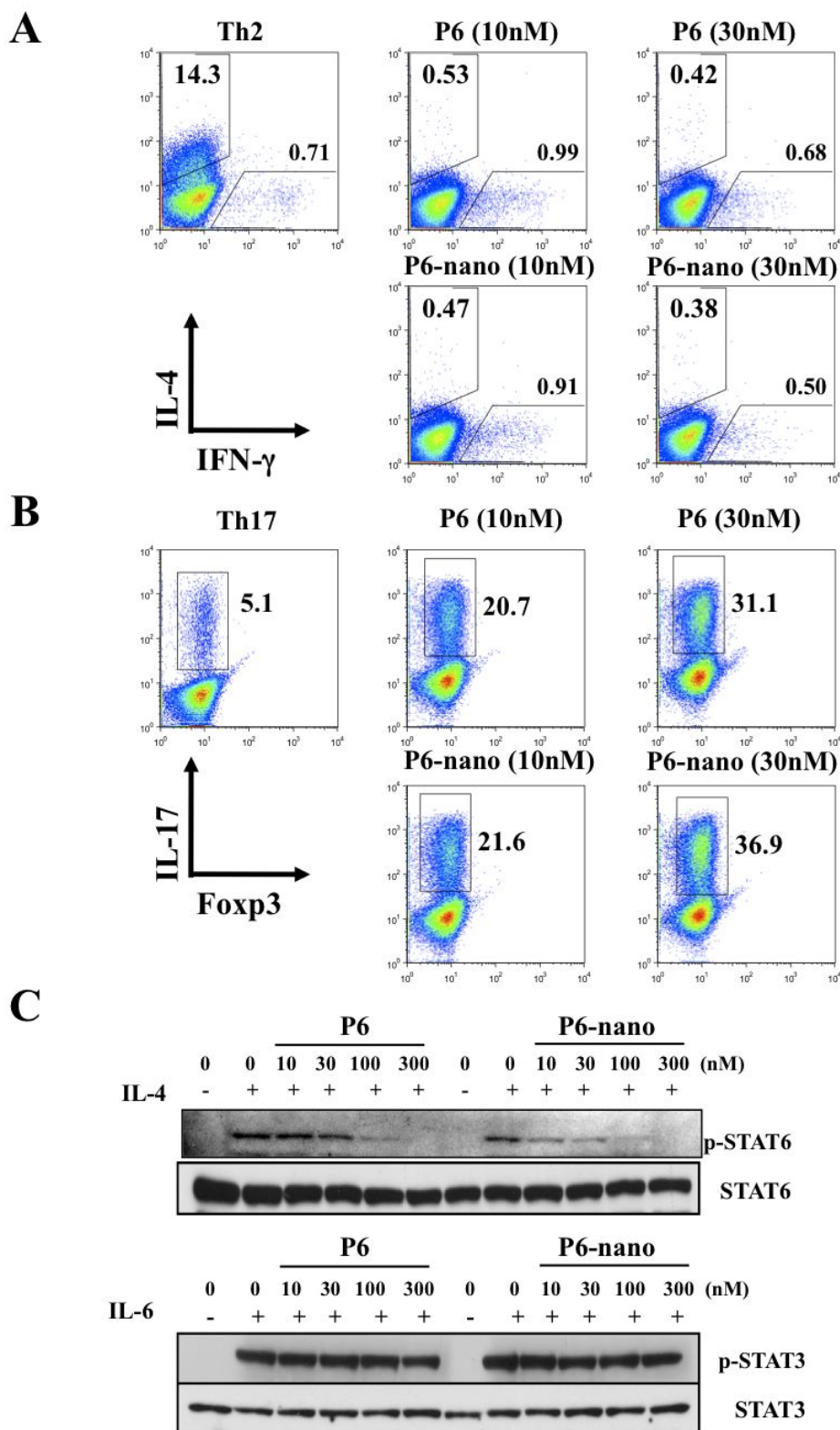
The authors have no financial conflicts of interest.

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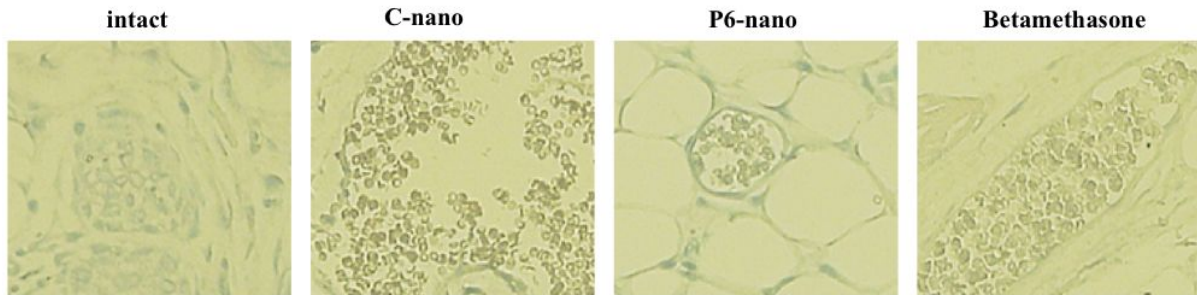
## Supplementary Fig. 1 P6 and P6-nano have a similar activity on Th differentiation



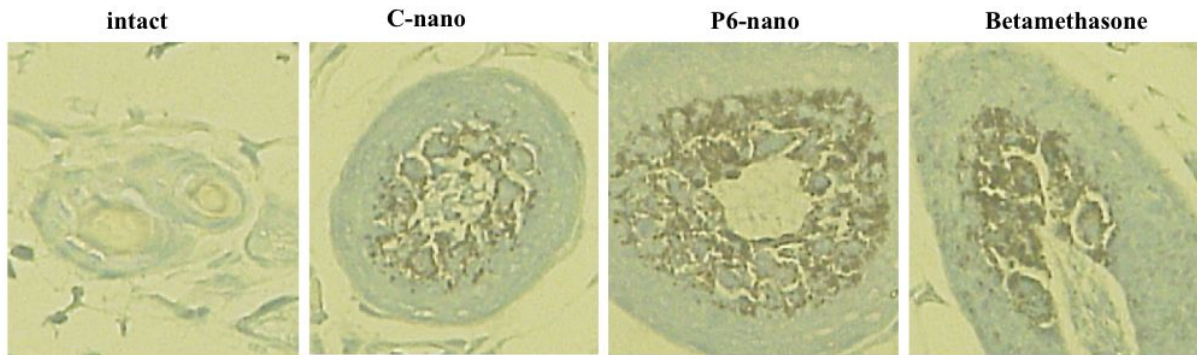
**Supplementary Figure 1** P6 and P6-nano have a similar activities on Th differentiation. A) Naïve CD4<sup>+</sup>T cells were cultured in Th2 or Th17 condition in the presence of 10 or 30 nM of P6 or P6-nano. After 2 days culture, T cells were restimulated with PMA and ionomycin and their intracellular cytokine production were measured. B) Naïve CD4<sup>+</sup>T cells were stimulated with plate bound anti-CD3 Ab and indicated cytokines in the presence or P6 or P6-nano at indicated concentrations. One hour later, cells were collected and a cytosolic fraction was prepared for STATs immunoblotting assay.

**Supplemental Fig. 2 P6 administration modifies phosphorylation of STAT1 and STAT3 in leukocytes and hair follicle keratinocytes *in vivo***

**A**

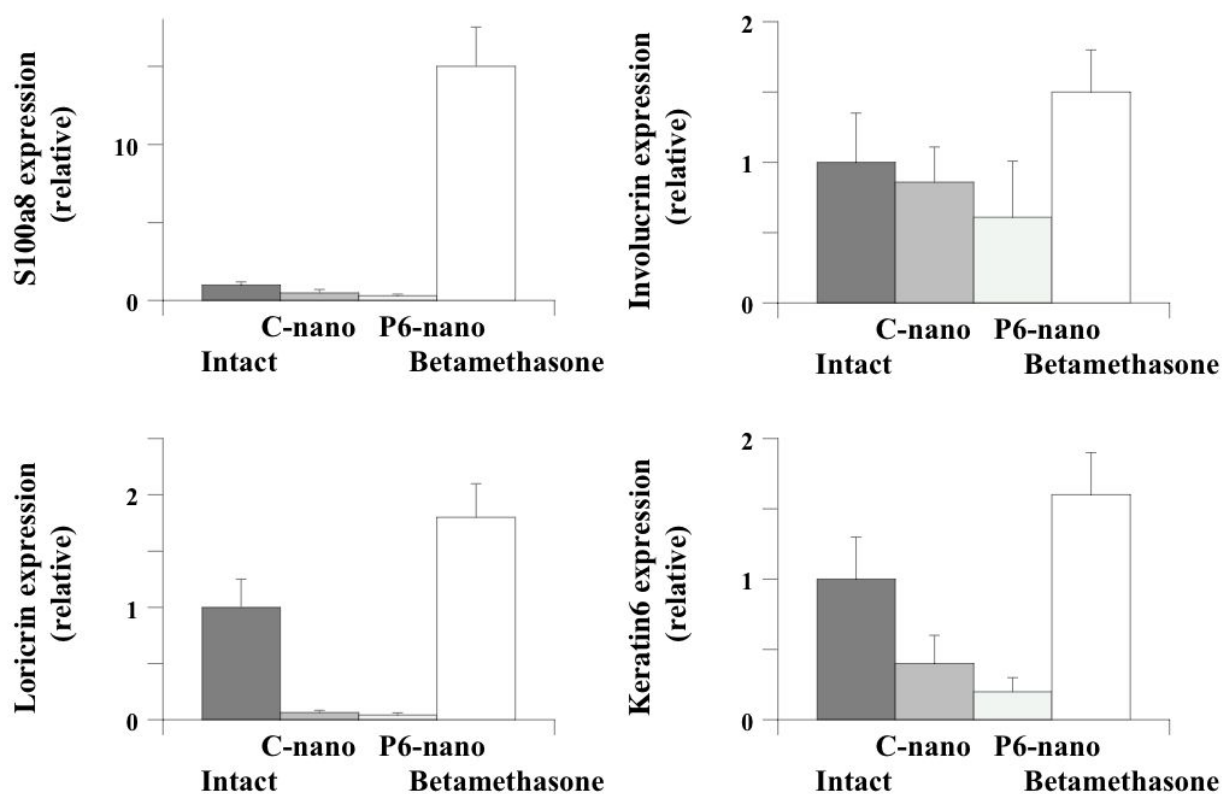


**B**



**Supplementary Figure 2** P6 administration modifies phosphorylation of STAT1 and STAT3 in leukocytes and hair follicle keratinocytes *in vivo*. Deparaffinized skin sections were stained with anti-phosphorylated STAT1 (A) or anti-phosphorylated STAT3 (B) antibodies. Magnification is x20. Typical histological observations are shown from 4 mice of each group.

### Supplementary Fig. 3 Effect of P6-nano on epidermal differentiation complex gene expression *in vivo*



**Supplementary Figure 3** Effect of P6-nano on epidermal differentiation complex gene expression *in vivo*. Total RNA was isolated from the skin lesions of intact or drug-treated mice, then mRNAs were measured by real-time RT-PCR analysis 4 weeks after onset of the disease. Results were presented relative to baseline expression in epidermal tissues from intact mice, setting as 1. Data represent mean  $\pm$  s.d. from triplicate experiments.